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(54) Title: DETECTION OF MUTATIONS OR VARIATIONS IN DNA

(57) Abstract

The invention provides method of detecting and/or characterising a nucleic acid, which comprises subjecting a test sample comprising the nucleic acid in double stranded form having first and second sequences hybridised to one another to electrophoresis under conditions of temperature which are varied for at least a portion of the time during which the electrophoresis is carried out, whereby migration rate is dependent on the degree of hybridisation between the first and second sequences, and detecting and/or characterising the nucleic acid on the basis of distance of migration. Also provided is an apparatus for carrying out the aforementioned method comprising means (20) to receive an electrophoresis medium (19) for receiving a sample to be subjected to electrophoresis, wherein in use, one or more components of the sample migrate across an operative part of the medium, means (14, 15) for applying to the medium an electrophoresis-inducing electrical voltage, and means (22, 24) for altering the temperature of at least the operative part of the medium whereby the temperature of the whole of the operative part of the medium is uniformly and progressively increased during at least a part of the period of time during which electrophoretic migration occurs.

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DETECTION OF MUTATIONS OR VARIATIONS IN DNA

This invention relates to methods and apparatus for the detection and/or characterisation of nucleic acids, especially nucleic acids having specific sequence characteristics.

In particular, this invention relates to the characterisation or identification of sequences, and to the detection or identification of sequence mutations or variations, in DNA, for example in PCR (polymerase chain reaction) products from a selected gene of interest from different individuals, and to a method and apparatus for achieving such characterisation, detection or identification. the invention is also applicable to the detection and/or characterisation of RNA and of DNA analogues such as PNA.

A known method of detecting and identifying such mutations or variations employs suitably labelled allele-specific oligonucleotides (ASO), typically 10-25 bases in length, which will pair base by base with the sequence of interest in a target strand which might, for example, be a PCR product from the region of interest in a DNA sample, for example a test sample of chromosomal DNA.

In prior art procedures, nucleic acids are often characterised or detected by probing with labelled oligonucleotide probes and detecting the presence or absence of binding under standard hybridisation conditions, including a selected temperature. Choice of temperature is important because an oligonucleotide probe which is annealed to a target strand will, under conditions of increasing temperature, dissociate from its target at or about some specific temperature. If there is a base pair mismatch with the target, the binding affinity will be lower and the oligonucleotide probe will dissociate from the target at a lower temperature than if no such mismatch is present.

It would be useful to be able to rely upon variations in dissociation temperature to detect and/or characterise nucleic acids, but this is not readily achievable in practice The reason, in part, is that the relationship

between degree of hybridisation and temperature when plotted graphically (the dissociation profile) is a curve rather than a step function. Accordingly, if the degree of hybridisation of a perfectly matched probe/target pair is compared to the degree of hybridisation of a probe target pair with a single mismatch, using temperature as the independent variable and percentage binding as the dependent variable, the result will be two curves, offset from one another. The separation between the two curves at a single temperature represents a "snapshot" of the two offset curves, but under normal conditions the separation of the curves may be insufficient to allow discrimination between matched and unmatched probe. Thus, it can be difficult or impossible to establish a condition where there is absolute distinction of binding (i.e. a condition under which the degree of binding of an oligonucleotide probe to one allele is distinguishable from the degree of binding to a second allele). Thus, a typical experiment with an "allele-specific" probe would involve the optimization of dissociation conditions and the choice of a temperature cut-off. Even when optimised, it may be difficult to distinguish between alleles.

The situation is rendered more complex in diploid organisms such as humans, where the sequence variation can be present in the heterozygote form, i.e. one chromosome has normal sequence and the other has mutant sequence, so that the melting profile becomes the sum of two displaced curves. Thus, standard methods for analysis tend to rely on a single temperature "snapshot" and thus fail to access the distinctive profiles of the full melting curves. It is therefore usually insufficient to use a single probe as a "normality" test, and the heterozygote form can only be detected by use of a second probe which is specific for the mutant sequence.

In conventional ASO analysis of this kind, the usual procedure is to attempt to detect probe which has not melted off and is still bound, after a washing operation at a predetermined "stringent" temperature. This bound probe is detected as still present during a subsequent measuring step: washed off mismatched oligonucleotide probe is not detected. For this

reason, it is necessary to use both normal and mutant probes in order to demonstrate heterozygosity conclusively.

It is an object of the present invention to provide an improved method and apparatus for the detection and/or characterisation of nucleic acids, especially nucleic acids having specific sequence characteristics.

According to one aspect of the present invention, there is provided a method of detecting and/or characterising a nucleic acid, which comprises subjecting a test sample comprising the nucleic acid in double stranded form having first and second sequences hybridised to one another to electrophoresis under conditions of temperature which are varied for at least a portion of the time during which the electrophoresis is carried out, whereby migration rate is dependent on the degree of hybridisation between the first and second sequences, and detecting and/or characterising the nucleic acid on the basis of distance of migration.

In one manner of carrying out the method of the invention, reliance is placed upon use of a detectable probe which migrates at a different (usually faster) rate when in free form than when hybridised to a target nucleic acid. Thus, preferably, the test sample of nucleic acid in double stranded form comprises a target strand and a detectable probe hybridised thereto, the rate of migration of the detectable probe being different when in free form than when hybridised to the target strand, whereby the distance of migration of the detectable probe is at least partly dependent upon the time interval that has elapsed following the time when the temperature reached a value at which dissociation of the probe and target occurred.

Preferably, in carrying out the process of the invention, a control sample comprising, in double stranded form said detectable probe hybridised to a control target strand, is also subjected to electrophoresis under comparable conditions to those applied to the test sample.

The method of the invention thus preferably comprises the steps of:

annealing to a target strand of said nucleic acid test sample, labelled oligonucleotide capable of hybridising with a sequence in the target strand,

subjecting the target strand, with labelled oligonucleotide annealed thereto to electrophoresis,

progressively increasing the temperature at which electrophoresis is carried out through a range of temperatures including a dissociation temperature at which dissociation of oligonucleotide from the target strand occurs,

continuing the electrophoresis during a time period after the said dissociation temperature has been reached, and thereby electrophoretically separating dissociated oligonucleotide, and

detecting or identifying sequence mutations or variations in the nucleic acid test sample based upon the position and displacement of dissociated oligonucleotide.

More specifically, the method may comprise the steps of:

annealing to target strands of DNA selected labelled oligonucleotides capable of pairing base by base with a sequence of interest in the target strands,

positioning of the target strands, with oligonucleotides annealed thereto, in an electrophoresis gel,

subjecting the electrophoresis gel and the samples therein to an electrophoresis-inducing electrical field,

in the presence of the electrical field, progressively increasing the temperature of the gel and samples through a range of temperatures including a dissociation temperature at which at least some of the oligonucleotides dissociate from the target strands to which they were annealed,

maintaining the electrical field during a time period after the said dissociation temperature has been reached, and thereby electrophoretically displacing from, substantially, their original positions those oligonucleotides which have dissociated from their target strands,

and measuring the positions and displacements of such oligonucleotides as a measure of the said time period since they dissociated

and hence of the temperature at which dissociation from their target strands occurred.

The invention according to a second embodiment thereof allows discrimination between allelic forms of a nucleic acid to be tested on the basis of different rates of migration of a nucleic acid species at different temperatures, while remaining in double stranded form.

Thus according to this aspect of the invention, there is provided a method as previously defined, wherein the test sample comprises a species of nucleic acid which remains substantially in double-stranded form for the duration of the time in which the electrophoresis is carried out, the migration rate of said species of nucleic acid is dependent on temperature, and the species of nucleic acid is detected and/or characterised on the basis of the distance of migration of the species of nucleic acid in double stranded form.

In carrying out the method of this aspect of the invention, the temperature may be either increased or decreased during the course of the electrophoresis.

Preferably the aforementioned species of nucleic acid migrates at relatively fast and slow rates at respective first and second temperatures and the electrophoresis is carried out under progressively changing conditions of temperature over a temperature range which includes said first and second temperatures. Preferably, the rate of change of temperature is programmed so as to be lower in the temperature range that includes said first and second temperatures than in temperature ranges above and/or below said range. Typically, said second temperature is higher than said first temperature, i.e. the temperature is increased during the electrophoresis, but as indicated, the invention may also be operated in a reverse manner, with the temperature being decreased.

In this embodiment, it is unnecessary to employ a labelled oligonucleotide probe. Thus, conveniently, the nucleic acid in double stranded form is unlabelled, i.e. the nucleic acid in double stranded form may comprise first and second unlabelled strands. Further, the nucleic acid in double stranded form may comprise first and second strands of substantially

equal length, i.e. the first and second strands can conveniently be not more than 20, preferably not more than 10 nucleotides different in length, and if desired may be of the same length. Alternatively, the nucleic acid comprises a single polynucleotide chain which adopts different conformational forms at different temperatures, for example it may consist of or include a hair-pin duplex.

The methods of the invention are especially useful for testing nucleic acid samples for the presence of mutations associated with genetic diseases. Thus, in this application, the detectable probe is adapted to hybridise with a target strand susceptible to allelic variation and the probe/target hybrids formed between different allelic forms of the target have different disassociation temperatures. Nucleic acid associated with a heterozygote may then be detected at different positions, each corresponding to one allelic form.

The invention, according to a further aspect thereof, provides apparatus comprising means to receive an electrophoresis medium for receiving a sample to be subjected to electrophoresis, wherein in use, one or more components of the sample migrate across an operative part of the medium, means for applying to the medium an electrophoresis-inducing electrical voltage, and means for progressively increasing or reducing the temperature of at least the operative part of the medium with time whereby the temperature of the whole of the operative part of the medium is uniformly and progressively increased or reduced during at least a part of the period of time during which electrophoretic migration occurs.

Preferably, the apparatus comprises means to receive a gel layer containing samples to be subjected to electrophoresis, means for applying to the gel layer an electrophoresis-inducing electrical voltage, and means for heating the gel layer through a range of temperatures while maintaining the gel temperature substantially uniform across the whole area of the layer or at least the operative part thereof.

It will be appreciated that the basis of the invention, in one of its preferred aspects, is that if a sample of an oligonucleotide probe bound to

a longer target strand is subjected to an electrophoretic force to cause it to migrate through a suitable electrophoresis gel, and the temperature of the gel and the sample is raised smoothly through a range which includes the temperature at which the oligonucleotide probe separates from its target strand, the oligonucleotide probe will migrate only slowly (or substantially not at all) while still bound to the relatively large target strand but more rapidly after becoming free from the target strand, with the result that the length of its migration track through the gel up to the time at which the electrophoresis force is removed affords a measure of the time interval since it dissociated from its target strand and thus of the temperature at which dissociation occurred.

If, in practice, dissociation of a given oligonucleotide probe from a given target strand takes place over a small range of temperature and/or over a small time interval rather than instantaneously when a specific temperature is reached, then the electrophoresis trace obtained from a sample will appear as a migration band or smear representing the complete dissociation or melting profile of the sample. If a sample contains target strands of which some provide a perfect match for the oligonucleotide probe but some provide a mismatch so that the oligonucleotide probe will melt off at a lower temperature, this will show as two separate bands or peaks of maximal intensity in the migration trace. In particular, a sample containing target strands from human or other diploid organisms, in which a sequence variation can occur in one or both of a pair of chromosomes, may provide such a double-peaked trace representing oligonucleotide probe which had annealed to, and then separated from, respectively, both the normal and the variant chromosomes.

In many instances of the method according to the invention, a selected oligonucleotide is annealed to at least some target strands which it matches perfectly and at least some further target strands with which it has at least a one-base mismatch, and wherein said range of temperatures includes a lower dissociation temperature at which the selected oligonucleotide dissociates from the said further target strands and a higher

dissociation temperature at which it dissociates from those target strands which it matches perfectly.

Preferably, the method of the invention is carried out using a thin layer of suitable electrophoresis gel (conveniently or preferably a high percentage polyacrylamide gel) supported on an electrically insulating substrate and provided with means for maintaining the gel temperature substantially uniform over the whole area of the layer, or at least its operative part, while increasing the temperature thereof; and preferably such a gel layer, having one face in contact with the supporting substrate, has formed in an exposed opposite face a plurality of wells in which a corresponding plurality of samples to be subjected to electrophoresis can be placed.

The method and embodiments of apparatus according to the invention will be more fully disclosed in and more completely understood from, the following description of examples thereof with reference to the accompanying drawings, in which;-

Figure 1, is an exploded schematic perspective view of an embodiment with a support rack thereof removed, of apparatus according to the invention for carrying out the method of the invention;

Figure 2 is a broken-away perspective view, on a larger scale, of the said support rack removed from the apparatus shown in Figure 1 and of substrate-mounted electrophoresis gel layers supported therein;

Figure 3 is a perspective view of a substrate-mounted gel layer which may be used instead of that shown in Figure 3;

Figure 4 is a representation of traces made on an electrophoresis plate as shown in Figure 3 by subjecting a variety of samples to electrophoresis by the method according to the first embodiment invention.

Figure 5 is a perspective view of another substrate-mounted gel layer which may be used instead of that shown in Figure 3;

Figure 6 shows a known PCR machine together with auxiliary components for adapting it to carry out the method of the present invention.

Figure 7 is a representation of traces made on an electrophoresis plate as shown in Figure 3 by subjecting a variety of samples to electrophoresis by the method according to the second embodiment of the invention.

The apparatus shown in Figure 1 comprises a tank 11 of electrically insulating material having mutually-opposite end walls 12 and 13 on the inner surfaces of which are provided electrodes 14 and 15 respectively, intended to have applied between them an electrophoresis-inducing voltage supplied by a voltage supply unit (not shown).

The tank 11 receives support rack 16, shown in Figure 2, in which are removably supported a plurality of electrophoresis plates 17 (also shown in Figure 3) each comprising a thin substrate 18 of electrically insulating material, such as glass, having adhered on its upper surface a layer of suitable gel material 19, such as high percentage polyacrylamide gel, in which to induce electrophoresis of samples inserted into wells 20 formed in the gel layer 19. After insertion of the samples, the gel layer 19 of each plate 17 may, if desired, be covered by a thin cover plate (not shown) of glass or the like prior to insertion of the plate 17 into the rack 16.

The tank 11 is also filled with a fluid such as Tris-borate-EDTA buffer (not shown) to a depth such that when the rack 16 is placed in the tank all the plates 17 supported in it are immersed in the fluid. Application of a voltage, suitably of 150 volts, between the electrodes 14 and 15 induces in the buffer fluid an electric field in the direction from one end wall of the tank to the other and thus lengthwise of the plates 17. If the rack 16 is inserted into the tank with the wells 20 of the plates 17 adjacent the end wall 13, the electrodes 14 and 15 will be connected as anode and cathode respectively in order to induce electrophoretic migration, of nucleic acids contained in samples inserted into the wells 20, along the gel layers 19, in the direction from the wells towards the remote ends of the plates 17, adjacent the end wall 12 of the tank 11.

The tank 11 is mounted upon a base 21 in which are housed infra-red lamps 22 serving to heat the buffer fluid in the tank 11. To improve the uniformity of this heating there is provided in the lower part of the tank 11

a plate 23 of suitable material such as Perspex and preferably black in colour which efficiently absorbs heat radiated from the lamps 11 and re-emits it by both radiation and conduction to heat the buffer fluid in which it is immersed. Below the plate 23, at the bottom of the tank 11, is a serpentine pipe 24 through which cold water can be circulated through inlet and outlet pipes 25 and 26 so that the buffer fluid may be cooled as well as heated. A valve (not shown) for control of water flow through the pipe 24 is contained within a housing 27 mounted on the tank 11 at its rear; and this housing also serves to mount a thermocouple 28 which projects downwardly into the buffer fluid and an impeller 29 driven by a motor (not shown) contained within the housing 27 to circulate the buffer fluid over the heating plate 23 and then over and between the electrophoresis plates 17. The housing 27 may also contain electrical circuitry (not shown) responsive to buffer fluid temperature as measured by the thermocouple 28 and to a pre-determined temperature-control programme, for controlling the supply of power to the lamp 22 so as to provide, over a pre-determined period, a controlled rise of temperature in the buffer fluid and thus in the plates 17 and their gel layers 19.

Typically, in use of the apparatus shown in Figure 1 to 3, samples to be investigated are introduced into some or all of the wells 20 of one or more plates 17, and the plates 19 are loaded into the rack 16 which is then placed in the tank 11 with the plates 17 immersed in the buffer fluid which fills the tank. Cold water at, say, 4°C, is then circulated through the serpentine pipe 24 for a period of, typically, one hour with the impeller 29 operative to circulate the buffer fluid so that it and the plates 17 and their gel layers 19 are all substantially at the same uniform low temperature of 4°C. After this initial cooling period, circulation of water through the pipe 24 is terminated, a d.c. voltage of, suitably, 150 volts is applied between the electrodes 14 and 15, with the electrode 15 adjacent to the wells 20 as cathode, and electrical power is supplied to the heater lamps 22 at a controlled rate to raise the temperature of the plate 23, buffer fluid, plates 17 and their gel layers 19 at a rate of 1°C per 5 minutes. This temperature-

raising regime, under the control of the preset program and monitored by means of the thermocouple 28, may suitably be continued until a temperature of 60°C has been reached. The electrophoresis voltage between the electrodes 14 and 15 is then switched off, as also the power supplied to the heater lamps 22 and the impeller 29, and the plates 17 may be removed for inspection of their electrophoresis traces (after any necessary processing to render them visibly or otherwise detectable) in known manner.

Figure 4 shows diagrammatically a typical resulting plate 17 of which seven of the wells 20 were filled with samples before subjecting the plate to electrophoresis under a controlled rising temperature regime in accordance with the invention, as described above. In this illustrated case, the samples in two of the wells, marked O, contained only oligonucleotide probe unbound to any target strand of DNA. In two further wells, marked NN, the sample contained oligonucleotide probe bound to target strands obtained from diploid chromosome fragments in which, however, both strands were normal so that the oligonucleotide probe was bound with a perfect match to all target strands. Two further wells, marked NM, contained samples prepared from a diploid species, e.g. human, in which one chromosome has a sequence of bases in a target strand fragment to which the selected oligonucleotide probe binds as a perfect match, whereas the corresponding sequence in a strand from the other chromosome contains at least one mismatch for the oligonucleotide probe. Such a mismatch, which may consist in a mutation of one or more bases of the sequence, or in the omission of one or more bases, results in the oligonucleotide probe being bound less tightly to the target strand from which, therefore, it becomes dissociated at a lower temperature.

The effect is reflected in the lengths of electrophoresis tracks made on the plate shown in Figure 4. The oligonucleotide probe in the samples in the wells marked O, being wholly unbound to any target strand, all begins to migrate along the plate as soon as the voltage is applied between electrodes 14 and 15, and continues to do so until the voltage is switched

off. The oligonucleotide probe is then detectable as a spot 30 at a distance l_1 from the corresponding well 20. The distance l_1 gives a measure of the time interval during which the electrophoresis voltage was applied between electrodes 14 and 15. Each of the other samples also contains unbound oligonucleotide probe which migrates during the whole time the voltage is applied and accordingly produces in each other trace spot at the same distance l_1 as the spots 30. In addition, however, each of these other samples also contains oligonucleotide probe which is bound to target strands and which therefore does not begin to migrate significantly until a temperature is reached at which it dissociates from the target strands and is thereby freed. In the wells marked NN, the target strands of the sample are all of normal sequence to which the oligonucleotide probe binds with perfect matching, and accordingly the oligonucleotide probe does not dissociate or melt off until a relatively high temperature, of, say, 45 or 50°C, has been reached. The freed oligonucleotide probe then migrates only during the relatively short time then remaining, to be detected as a spot 31 at a correspondingly short distance of only (l_1-l_2) represents the distance which the "never bound" oligonucleotide probe melted off and was free to migrate. In the case of the wells marked NM, the samples contained unbound oligonucleotide probe and oligonucleotide probe bound to target strands some of which show normal base sequence for which the oligonucleotide probe is a perfect match but some of which contain a base sequence mismatched for the oligonucleotide probe which therefore melts off at a lower temperature. Accordingly, the traces from the wells marked NM show spots corresponding to the spots 30 and 31, but also spots 32 at a distance l_3 (greater than l_2) from the wells 20, the spots 32 being due to oligonucleotide probe melted off from target strands to which it was not perfectly matched and thus at a lower temperature, reached earlier, than the melt-off temperature for perfectly matched oligonucleotide probe. Finally, the well marked MM contained target strands all of which exhibited a mismatch for the oligonucleotide probe and the resulting trace therefore

contains spots corresponding to the spots 30 and 32, but no spot corresponding to the spots 31.

As illustrated in Figure 4, the trace features referred to above as "spots" 30, 31 and 32 are not entirely sharply defined but are more or less elongated in the direction of migration, with a region of maximum intensity preceded and/or followed by regions of lesser intensity. In the case of the spots 30, representing oligonucleotide probe which had never been bound, this spread of the spots represented the statistical diffusive nature of the electrophoretic migration. This is also a factor in the configuration of the spots 31 and 32; but with these another factor is also involved. The spots 31 and 32 represent oligonucleotide probe whose electrophoretic migration commenced only after the oligonucleotide probe dissociated or melted off from the target strand to which it was bound originally, and the totality of this melting off does not occur instantaneously the moment some particular critical temperature is reached. Rather, it takes place over a period of time, usually at a temperature-dependent rate, once some adequate temperature has been reached, or over a range of temperatures in a rising-temperature regime such as has been described above. The elongate spots 31 and 32, therefore, represent temperature- and time-dependent "melting profiles" for the dissociation of a selected oligonucleotide probe from different target strands to which it has been bound. It will be appreciated that, even when elongated spots in a trace merge with one another to some extent, as depicted in the traces from the wells marked NM, their regions of maximum intensity are usually recognisable and distinguishable from one another without difficulty.

The plates 17 shown in Figures 2 and 3 each have a single row of wells 20 near one end, with almost the whole length of each plate available for producing long electrophoretic traces. If shorter traces are acceptable, additional rows of wells may be provided, each extending across the width of the plate and spaced from one another along its length so as to divide the surface into separate regions to accommodate the (shorter) traces from the respective rows of wells. If the samples to be investigated by means of the

present invention will have been prepared in large numbers in wells arranged in a standard microtitre array, then, to facilitate mechanical transfer of all the samples simultaneously to wells in the gel layer of an electrophoresis plate, the wells 20 in the gel layer 19 of the plate 17 may, as shown in Figure 5, be arranged in an array 33 on a lattice identical with that of the standard microtitre array; and in order to make an adequate electrophoresis track length available, extending from each well, the array 33 may, as disclosed in UK Patent Application No. 9324901.9, and as shown in Figure 5, be set at an angle to the lengthwise direction of the plate 17 and thus to the direction of the electrophoresis-inducing electrical field which will be applied along it, so that a track 34 extending from a well 20 in on row of the array can pass between two mutually adjacent wells in the next row and, preferably, a further pair of mutually adjacent wells in the row after that before encountering a well in a still more distant row.

The apparatus shown in Figure 1 and 2 is dedicated apparatus constructed specifically for carrying out the method of the present invention; but apparatus already exists which may be modified or converted for use according to the invention, particularly when only limited or small-scale use is contemplated. For example a known, standard, PCR machine as shown in Figure 6 comprises a thermoconductive block 35, formed with wells 36 and mounted on a base housing 37 which includes controlled heating means (not shown) for taking the block 35 (and samples inserted in its wells 36) through a predetermined temperature cycle as required for subjecting the samples to the PCR process in known manner.

An adaptor tray 38, with good thermal conductivity, is formed with "feet" 39 such that the tray and its feet are a close fit over the block 35 and within the wells 36. When the tray 38 is fitted over the block 35 with its feet 39 nested in the wells 36, the heated block 35 maintains the upper surface of the tray at a substantially uniform temperature which varies time-wise with that of the block. A shallow tank 40, corresponding to the tank 11 of Figure 1, is provide to receive a buffer liquid in which one or more electrophoresis plates 17 can be immersed and subjected to an electric field

due to a voltage applied between electrodes 41 and 42 with which the tank 40 is provided. The tank may be provided with a thermally insulating lid 43 of, say, expanded polystyrene, which may be formed to allow a temperature probe 44 to dip into the buffer fluid in the tank 40. The probe 44, preferably, is that, forming part of the PCR machine, which would be positioned in one of the wells 36 of the block 35 in use of the machine for its normal purpose, for monitoring the temperature of the block and providing a feedback signal to the temperature-profile control of the heating means house in the base 37. This control may be pre-set so as to provide the desired temperature-ramp profile for carrying out the method of the present invention substantially as described above with reference to the apparatus shown in Figures 1 and 2.

It will be understood from the foregoing description that the essence of this aspect of the invention is not only to obtain information relating to perfect-match probe binding events (as in the known use of such techniques) but more generally, by combining the probe binding with electrophoresis of the samples while applying a controlled rise in temperature, to display complete melting profiles of the samples, i.e. to obtain information about the binding events which are not perfectly matched as well as those that are. This aspect of the invention may be employed in any of the fields in which oligonucleotide probe binding is or can be utilised, for example in the determination of sequence variation at specific sites, using ASOs, or in the technique known as "sequencing by hybridisation", in which arrays of many oligonucleotides are used to determine wide ranges of sequence variation or to determine unknown sequences de novo.

The production of a complete profile of oligonucleotide probe melting, in contrast to known ASO methods which look only at the oligonucleotide probe still bound, in a temperature "snapshot" of the profile, means that, in use with a heterozygote, an ASO representing the normal sequence (rather than a mutant mismatch) generates a much greater amount of information since the profile displays three bands or smears in order of decreasing migration: firstly the "never bound" oligonucleotide probe which migrates as

oligonucleotide probe during the whole duration of the run; secondly, oligonucleotide probe released at a lower temperature because it had a mismatch with mutant PCR target strands to which it had been bound; and finally, oligonucleotide probe released at a high temperature because it had been annealed with perfectly cognate target strands in the PCR product. The greater output of information derived from use of the invention is believed to be novel, and makes the invention of high utility in practical terms. It is believed to be the only method using ASO which looks at the "freed" as well as the "still-bound" oligonucleotide probe; and the use of electrophoresis rather than bulk flow for separating freed oligonucleotide probe from the target strand means that there is no dilution of signal, which is another very useful feature. Other features which are actually or potentially useful and advantageous include:

a) the spatial resolution and display of both perfect and imperfect probe binding in a mixture would have combinatoric potential limited only by the number of theoretical plates in the gel;

b) the probe/target annealing is entirely in mobile (liquid) phase, thus avoiding the distortions and lesser access of solid phase anchorage which may artefactually flatten the melting curve;

c) it does not matter if the target happens also to be labelled, because of the eventual spatial resolution from oligonucleotide probe signal;

d) the early separation of bound probe from mismatched mutant target effectively selects for favourable detection of the mutant. There will be little background signal in this region of the gel. A pool of probes recognising adjacent regions might be used: if there is any variation, a component of the signal will be found in this region of the gel, otherwise it will not;

e) apart from obtaining sufficient signal, interpretation is not dependent on the concentration of the target strands;

f) in heterozygotes, the reannealing "bubble" of PCR strands with a mismatch, may favour binding and hence favour the signals obtained in such samples;

g) the gel shift of the more mobile species (e.g. the oligogonucleotide probe) by the larger, less mobile PCR target, is far greater than the reciprocal gel shift, and in high percentage gels the mobility of PCR targets approaches zero.

Referring to Figure 7, DNA samples were obtained from four patients who were known to exhibit heterozygosity for mutations in the low density lipoprotein receptor gene. The nature of the mutations for each patient were known as a result of previous extensive analyses, including sequence determination of the mutant gene to determine the precise base changes responsible for the mutations.

The nucleic acid samples consisted of a 268 base pair PCR product with an additional 50 base pair "GC clamp" attached at its 5'end.

An estimated 0.4pmoles of DNA were applied to the wells of the electrophoresis gel and subjected to electrophoresis with an applied current of 0.5A over a period of 1 hour. During this time, the temperature of the gel was raised from 65 to 70°C.

On completion of the electrophoresis, the gel was stained using ethidium bromide and examined under a UV light.

The results, depicted schematically in Figure 7, show that for the controls in lanes A and F, only a single band 70 was visible on the gel. Corresponding distinct bands 70' are visible in tracks B, C and D and as part of band 79 in track E.

In the tracks B, C, D and E heteroduplexes which melted to form less mobile molecular species at an earlier stage during the electrophoresis (and consequently at a lower temperature) were observed nearer to the origin (bands 72, 76, 78 and 80).

In addition, homoduplexes in tracks B and D were observed as slightly faster migrating species as bands 71 and 77. It is believed that the mutant homoduplexes migrated faster because of their greatest stability than the normal (wild type) homoduplex. The mutations in the nucleic acid samples in lanes B and D consisted of the substitution of a GC base pair in the mutant for an AT base pair in the normal nucleic acid. The greatest stability

conferred by the presence of an additional GC base pair resulted in a more stable homoduplex that migrated as a slightly faster migrating species.

The nucleic acid in lane C resolved into four bands and the mutant homoduplex is believed to be the second fastest migrating band 74 of the ladder of four bands. This species is less stable than the normal homoduplex because it has an AT base pair instead of the normal GC base pair. Bands 75 and 76 represent the less stable heteroduplex forms.

In track E the band 70'/79 consisted of a band 70' which corresponded to bands 70 in tracks A and E and another homoduplex with a substantially idntical migration rate.

It is apparent from the results illustrated in Figure 7 that the method of the invention, according to the second embodiment thereof, allows the identification and characterization of mutant nucleic acids in a procedure which does not involve the use of labelled olinucleotide probes.

CLAIMS

1. A method of detecting and/or characterising a nucleic acid, which comprises subjecting a test sample comprising the nucleic acid in double stranded form having first and second sequences hybridised to one another to electrophoresis under conditions of temperature which are varied for at least a portion of the time during which the electrophoresis is carried out, whereby migration rate is dependent on the degree of hybridisation between the first and second sequences, and detecting and/or characterising the nucleic acid on the basis of distance of migration.

2. A method according to Claim 1, wherein the test sample of nucleic acid in double stranded form comprises a target strand and a detectable probe hybridised thereto, the rate of migration of the detectable probe being different when in free form than when hybridised to the target strand, whereby the distance of migration of the detectable probe is at least partly dependent upon the time interval that has elapsed following the time when the temperature reached a value at which dissociation of the probe and target occurred.

3. A method according to Claim 2, wherein the rate of migration of the detectable probe is greater when in free form than when hybridised to the target strand,

4. A method according to Claim 2 or Claim 3 wherein the detectable probe is capable of hybridising with a target strand susceptible to allelic variation and the probe/target hybrids formed between different allelic forms of the target have different dissociation temperatures.

5. A method according to Claim 4 wherein nucleic acid associated with a heterozygote is detected by the presence of detectable probe at different positions, each corresponding to one allelic form.

6. A method according to any preceding claim wherein a control sample comprising, in double stranded form said detectable probe hybridised to a control target strand, is also subjected to electrophoresis under comparable conditions to those applied to the test sample.

7. A method according to any preceding claim wherein the nucleic acid to be identified comprises DNA.

8. A method according to any preceding claim wherein the electrophoresis is carried out on an electrophoresis gel.

9. A method according to any preceding claim wherein the detectable probe has a length of 10 to 25 bases.

10. A method of detecting or identifying sequence mutations or variations in a nucleic acid test sample, comprising the steps of:

annealing to a target strand of said nucleic acid test sample, a labelled oligonucleotides capable of hybridising with a sequence in the target strand,

subjecting the target strand, with labelled oligonucleotide annealed thereto to electrophoresis,

progressively increasing the temperature at which electrophoresis is carried out through a range of temperatures including a dissociation temperature at which dissociation of oligonucleotide from the target strand occurs,

continuing the electrophoresis during a time period after the said dissociation temperature has been reached, and thereby electrophoretically separating dissociated oligonucleotide, and

detecting or identifying sequence mutations or variations in the nucleic acid test sample based upon the position and displacement of dissociated oligonucleotide.

11. A method of detecting or identifying sequence mutations or variations in DNA, comprising the steps of:

annealing to target strands of DNA selected labelled oligonucleotides capable of pairing base by base with a sequence of interest in the target strands,

positioning of the target strands, with oligonucleotides annealed thereto, in an electrophoresis gel,

subjecting the electrophoresis gel and the samples therein to an electrophoresis-inducing electrical field,

in the presence of the electrical field, progressively increasing the temperature of the gel and samples through a range of temperatures including a dissociation temperature at which at least some of the oligonucleotides dissociate from the target strands to which they were annealed,

maintaining the electrical field during a time period after the said dissociation temperature has been reached, and thereby electrophoretically displacing from, substantially, their original positions those oligonucleotides which have dissociated from their target strands,

and measuring the positions and displacements of such oligonucleotides as a measure of the said time period since they dissociated and hence of the temperature at which dissociation from their target strands occurred.

12. A method according to Claim 1 wherein the test sample comprises a species of nucleic acid which remains substantially in double-stranded form for the duration of the time in which the electrophoresis is carried out, the migration rate of said species of nucleic acid is dependent on temperature, and the species of nucleic acid is detected and/or characterised on the basis

of the distance of migration of the species of nucleic acid in double stranded form.

13. A method according to Claim 12 wherein the species of nucleic acid migrates at relatively fast and slow rates at respective first and second temperatures and the electrophoresis is carried out under progressively changing conditions of temperature in time over a temperature range which includes said first and second temperatures.

14. A method according to Claim 13 wherein said second temperature is higher than said first temperature.

15. A method according to Claim 13 or Claim 14 wherein the rate of change of temperature is programmed so as to be lower in the temperature range that includes said first and second temperatures than in temperature ranges above and/or below said range.

16. A method according to any of Claims 1 and 12 to 15 wherein nucleic acid in double stranded form is unlabelled.

17. A method according to Claim 16 wherein said nucleic acid in double stranded form comprises first and second unlabelled strands.

18. A method according to any of Claims 1 and 12 to 17 wherein said nucleic acid in double stranded form comprises first and second strands of substantially equal length.

19. A method according to Claim 18 wherein the first and second strands are not more than 20, preferably not more than 10 nucleotides different in length..

20. A method according to Claim 19 wherein the first and second strands are of the same length.

21. A method according to any of Claims 1 and 12 to 17 wherein said nucleic acid comprises a single polynucleotide chain which adopts different conformational forms at different temperatures.

22. A method according to Claim 21 wherein the nucleic acid comprises a polynucleotide chain in the form of a hair-pin duplex.

23. Apparatus for carrying out the method of any preceding claim, such apparatus comprising means to receive an electrophoresis medium for receiving a sample to be subjected to electrophoresis, wherein in use, one or more components of the sample migrate across an operative part of the medium, means for applying to the medium an electrophoresis-inducing electrical voltage, and means for increasing or reducing the temperature of at least the operative part of the medium whereby the temperature of the whole of the operative part of the medium is uniformly and progressively increased or reduced during at least a part of the period of time during which electrophoretic migration occurs.

24. Apparatus according to Claim 23 comprising means to receive gel layer containing samples to be subjected to electrophoresis, means for applying to the gel layer an electrophoresis-inducing electrical voltage, and means for heating the gel layer through a range of temperatures while maintaining the gel temperature substantially uniform across the whole area of the layer or at least the operative part thereof.

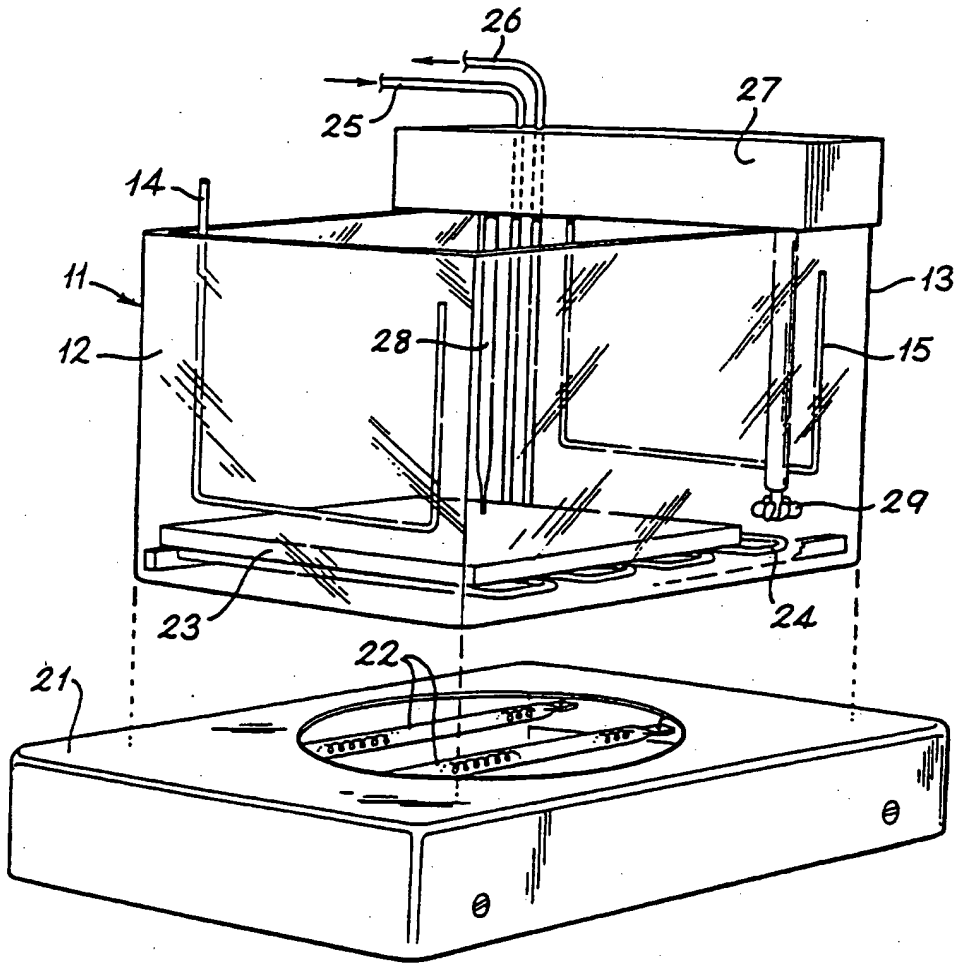

Fig. 1

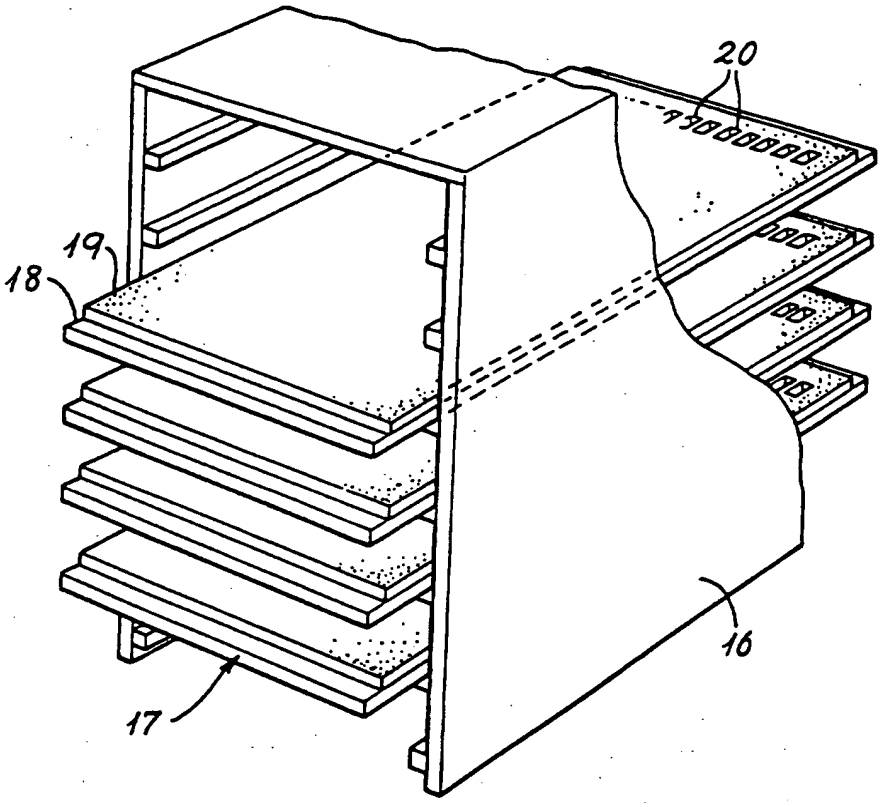

Fig. 2

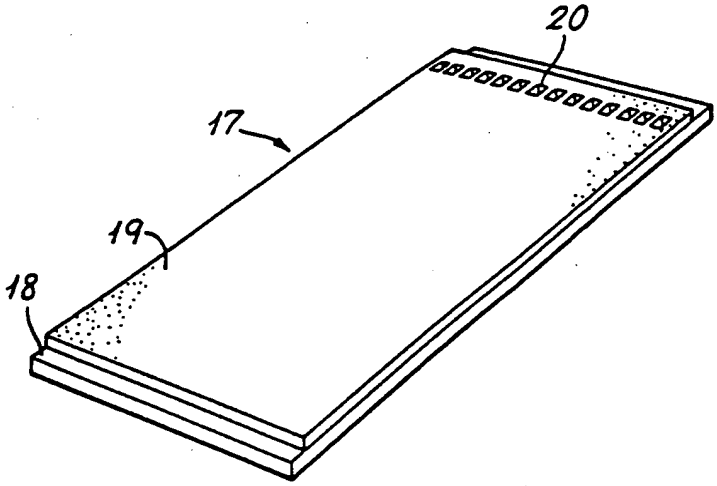

Fig. 3

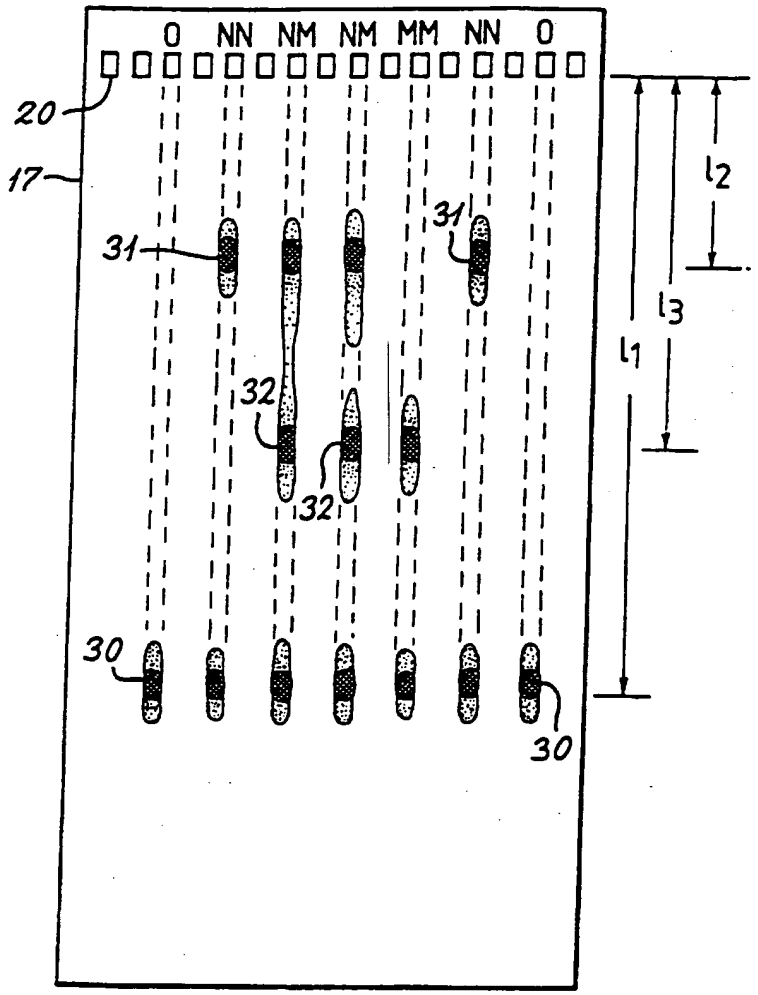

Fig. 4

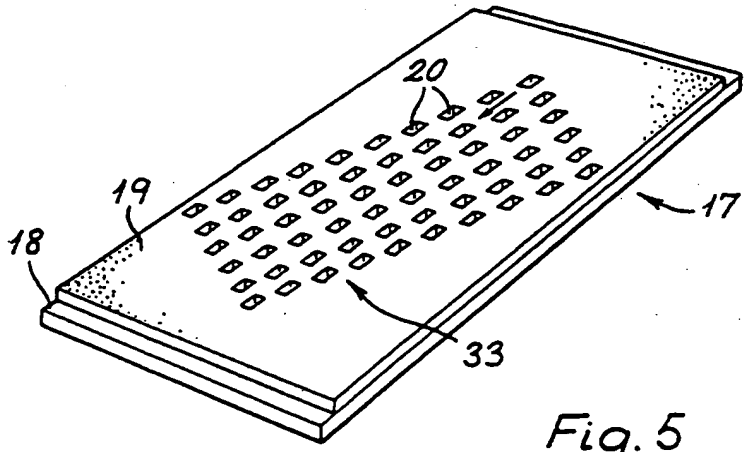

Fig. 5

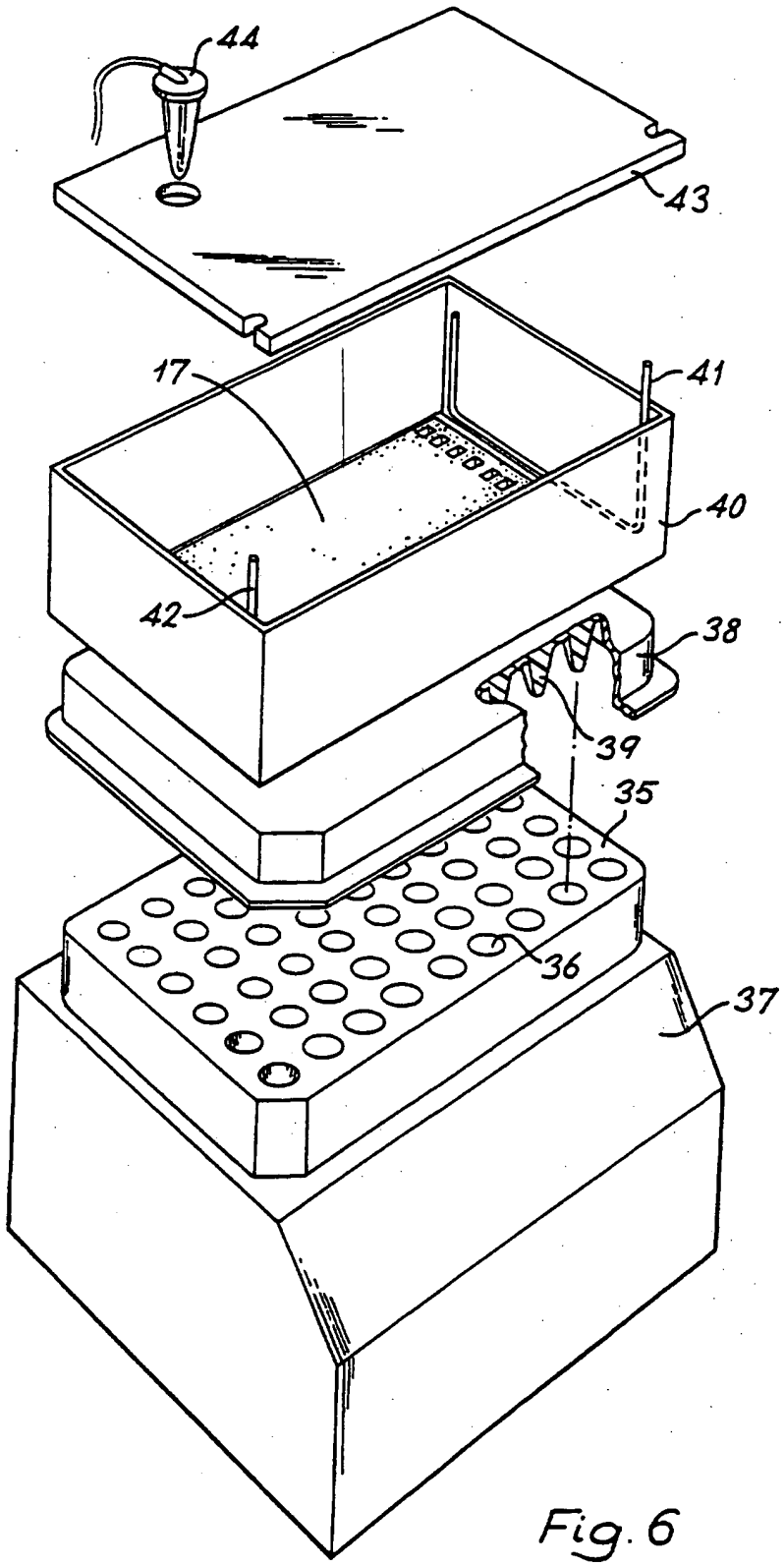

Fig. 6

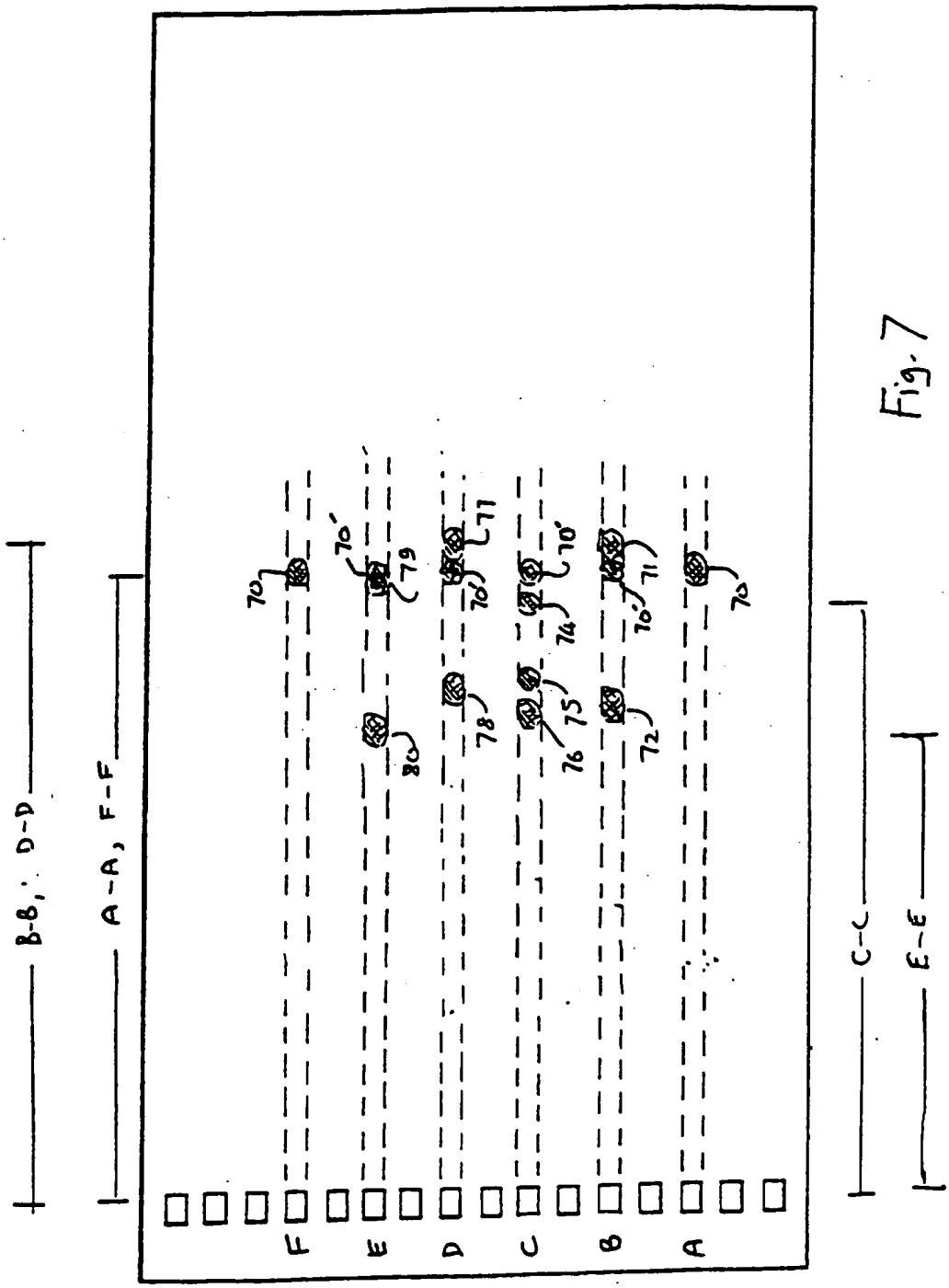

Fig. 7

INTERNATIONAL SEARCH REPORT

national Application No
PCT/GB 96/00283

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N27/447 G01N27/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 02815 (DIAGEN INSTITUT FÜR MOLEKULARBIOLOGISCHE DIAGNOSTIK GMBH) 7 March 1991 see the whole document ---	1-24
X	WO,A,93 16194 (DIAGEN INSTITUT FÜR MOLEKULARBIOLOGISCHE DIAGNOSTIK GMBH) 19 August 1993 see the whole document ---	1-24
A	WO,A,91 00925 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 24 January 1991 see the whole document ---	1-24
A	FR,A,2 686 621 (APPLIGENE SA) 30 July 1993 see the whole document ---	1-24
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
28 May 1996	27.06.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUTATION RESEARCH, vol. 285, 1993, pages 124-144, XP000443992 COTTON, R.: "current methods of mutation detection" see page 127 - page 128 ---	1-24
P,A	WO,A,95 21268 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 10 August 1995 see the whole document ---	1-24
T	NUCLEIC ACIDS RESEARCH, vol. 23, no. 13, 11 July 1995, pages 2404-12, XP002003146 DAY, IAN. ET AL: "electrophoresis for genotyping: temporal thermal gradient gel electrophoresis for profiling of oligonucleotide dissociation" see the whole document ---	1-24
T	DATABASE WPI Week 9612 Derwent Publications Ltd., London, GB; AN 96-107596 XP002003149 "electrophoresis time temperature with timed temperature variation" & ES,A,2 080 690 (UNIV ALCALA DE HENARES) , 1 February 1996 see abstract -----	1-24

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

International application No.

INTERNATIONAL SEARCH REPORT

PCT/GB96/00283

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 23 and 24
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

CLAIMS 23 AND 24 CONCERN AN APPARATUS PER SE. THE SEARCH DIVISION CONSIDERS THAT A COMPLETE SEARCH FOR SUCH AN APPARATUS IS NOT ECONOMICALLY FEASIBLE AND THEREFORE IT HAS BEEN RESTRICTED TO THOSE CLASSES COVERING THE USE OF T HE APPARATUS OF CLAIMS 23 AND 24 FOR CARRYING THE METHODS OF CLAIMS 1-22.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

national Application No
PCT/GB 96/00283

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9102815	07-03-91	DE-A-	3927467	21-02-91
		DE-A-	4006974	12-09-91
		EP-A-	0486580	27-05-92
		JP-T-	5503351	03-06-93
WO-A-9316194	19-08-93	DE-A-	4234086	12-08-93
		EP-A-	0581953	09-02-94
WO-A-9100925	24-01-91	US-A-	5045450	03-09-91
		CA-A-	2062974	14-01-91
		DE-D-	69008179	19-05-94
		DE-T-	69008179	17-11-94
		EP-A-	0482078	29-04-92
		JP-T-	4506456	12-11-92
FR-A-2686621	30-07-93	CA-A-	2128418	05-08-93
		EP-A-	0581938	09-02-94
		WO-A-	9315223	05-08-93
		JP-T-	7506483	20-07-95
WO-A-9521268	10-08-95	NONE		

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